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STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXIN

Annual Report

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May 26, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4245

University of Wisconsin
Madison, Wisconsin 53706

SECURITY CLASSIFICATION OF THIS PAGE

AD-A202 844

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Food Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) University of Wisconsin-Madison Madison, Wisconsin 53706	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-84-C-4245
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3MI-62770A871; TASK NO. AB; WORK UNIT ACCESSION NO. 362

11. TITLE (Include Security Classification)

(U) STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXINS

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 9/15/86 TO 9/14/87	1988, May, 26	6

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	01		Secondary structures, circular dichroism, tyrosine ionization, nicking, light and heavy chains, lipid bilayer surface, FPLC, types A, B and E neurotoxins, RA 1
06	20		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Analysis of the secondary structures of the isolated light and heavy chains of type A neurotoxin (NT) by circular dichroism showed that the two subunit chains did not undergo significant structural changes when separated from each other. Nicking of the single chain type E into the dichain form brought about conformational changes that were detected based on ionization of tyrosine residues. Both the light and heavy chains of types A, B and E NTs were found to attach to the lipid bilayer surface. Parameters of Fast Protein Liquid Chromatography of type A, B and E NTs were developed.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary F. Bostian	22b. TELEPHONE (Include Area Code) 301/663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

The secondary and tertiary structures of the neurotoxin (NT) and the state of tyrosine and tryptophan residues were determined using different techniques, such as circular dichroism, fluorescence spectroscopy and UV-difference spectroscopy. In the dichain type A NT the light and heavy chains appear to retain two quasi-independent structural domains although connected by -S-S- bonds. In other words the secondary structures of the subunit chains do not significantly change when they are separated after breaking the -S-S- and noncovalent bonds linking them. Examination of the ionization of phenolic groups of tyrosine residues of dichain type A, E and single chain type E NT has detected conformational changes resulting from nicking the single chain to the dichain protein; thus dichain type E NT appears more like dichain type A NT. Both the light and heavy chains of types A, B and E NTs were found to attach to the lipid bilayer surface. Parameters of Fast Protein Liquid Chromatography of types A, B and E NTs were developed.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, Revised 1985).

BODY OF REPORT

Clostridium botulinum produces neurotoxin (NT) in antigenically distinct forms called types A, B, C_1, D, E, F and G. In general, a pure culture produces one immunological type of NT, e.g. type A NT is produced by type A strain. The NT is a single chain (unnicked), a dichain (nicked) molecule or a mixture of single and dichain molecules. The two chains are held together by at least one -S-S- bond and by weak non-covalent bonds. The mol. wt. of heavy (or H) chain, ~100,000, is twice the weight of the light (or L) chain. All NT types are of mol. wt. ~150,000 (1,2). The single chain molecule is nicked endogenously into the dichain form in cultures of some (e.g. type A) but not in all types (e.g. type E). Mild trypsinization of any single chain NT results in its nicking. The separated individual chains appear non-toxic. The amino acid compositions of dichain NT types A, B and E, their H and L chains and the partial amino acid sequences of these three NTs are now known (3). Nothing is known about their conformation (secondary and tertiary structures).

In our studies of the antigenic/immunogenic characteristics of the NT, two kinds of antigenic determinants are being considered: i) "A segmental (continuous) site exists wholly within a continuous segment of amino acid sequence. ii) An assembled topographic site consists of amino acid residues far apart in the primary sequence but brought together in the surface topography of the native protein by the way it folds in three dimensions" (4).

Identification of the amino acid residues that are critical for immunochemical reactivity were examined by selective modification of certain amino acid residues and then determining their role in the antigenic structure. Chemical modification of amino acid residues might have altered the conformation of the NT which in turn directly or indirectly might change the antigenic/immunogenic reaction. No data was available on the conformation of the NTs therefore we expanded our studies in this area using UV difference spectrophotometry, circular dichroism and fluorescence techniques.

Interactions between the two subunits of the NT and model lipid surfaces were examined to understand the hydrophobic/hydrophilic sites on the surface of the NT and therefore indirectly the immunogenic/antigenic sites on the NT.

We utilized an automated modern high resolution ion exchange chromatographic system to distinguish three antigenically different NT types from a mixture. The resolution achieved for the first time was due to differences in charges on the protein surfaces probably brought about by the conformational differences between these NTs.

These separate, independent studies are presented as four sections with their experimental methods, results, discussions and conclusions.

1. pH INDUCED DIFFERENCE SPECTRA OF BOTULINUM NEUROTOXIN TYPES A, B AND E:

To understand the role of amino acid residues in the structure and function of the neurotoxin (NT) we examined the state of tyrosine residues based on ionization of phenolic groups and UV difference spectra.

The alkaline pH induced difference spectra (270-310 nm) of three antigenically distinct forms of the botulinum NT types A, B and E were examined. When isolated from the cultures of Clostridium botulinum, type A NT is a fully toxic dichain (nicked) protein, type E is a mildly toxic single chain (unnicked) protein, and type B NT is a mixture of single and dichain proteins and near fully toxic. Trypsin nicks the single chain protein to the dichain and increases its toxicity (up to about 100 fold in type E). A strong difference spectrum peak at ~296 nm was found when types A, B or E NTs were in the alkaline pH region. This peak was not observed at pH 4.0. For types A and B NTs plots of difference absorptivity vs. pH were simple sigmoidal curves. The pK of phenolic moieties of tyrosine residues in both proteins were 10.9. Nearly all tyrosine residues in both proteins were ionized. The single chain type E, unlike type A and B NT, yielded a two step titration curve and pK values 11.3 and less than 7.5; about 60% of the total tyrosine residues present were ionized. The two step titration curve was not observed when the single chain protein was nicked with trypsin to the dichain type E NT. The titration curve of dichain type E NT, although complex, was more like those of type A and B NT.

2. CONFORMATION OF LIGHT AND HEAVY CHAINS OF TYPE A NEUROTOXIN:

The secondary and tertiary structural features of botulinum NT serotype A, a dichain protein (M_r 145,000), and its two subunits, the heavy (H) and light (L) chains (M_r 97,000 and 53,000, respectively) were examined using circular dichroism and fluorescence spectroscopy. Nearly 70% of the amino acid residues in each of the three polypeptide preparations were found in ordered structure (sum of α helix, ß-sheet and ß turns). Also, the α-helix, ß-sheet, ß-turns and random coil contents of the dichain NT were nearly equal to the weighted mean of each of these secondary structure parameters of the L and H chains; e.g., sum of α helix of L chain (22%) and H chain (18.7%), as weighted mean, 19.8% was similar to that of NT (20%). (See table below.) These agreements suggested that the secondary structures of the subunits of the dichain NT do not significantly change when they are separated as isolated L and H chains. Fluorescence emission maximum of L chain, 4 nm less (blue shift) than that of H chain, suggested relatively more hydrophobic environment of fluorescent tryptophan residue(s) of L chain. Tryptophan fluorescence quantum yields for L chain, H chain and the NT, 0.072, 0.174 and 0.197, respectively, suggested that: i) an alteration in the micro-environment of the tryptophan residues was possibly caused by interactions of L and H chain subunits of the NT; and ii) quantum yields for L and H chains were altered when they are together as subunits of the NT.

Secondary structural parameters of L chain, H chain and the A NT calculated from their respective far UV circular dichroic spectra between 240 and 200 nm.

Proteins	α-helix, %	ß-sheet, %	ß-turn, %	Random coil %
A NT	20.00*	37.50	15.25	27.25
L chain	22.00	27.50	18.75	31.75
H chain	18.75[a]	40.00	13.00	28.25
Weighted mean**	19.83	35.83	14.92	29.42

**Calculated as (1 x L chain + 2 x H chain)/3 because H chain is twice the size of the L chain.

3. INTERACTION OF THE NEUROTOXIN WITH LIPID BILAYER SURFACE:

The interaction of botulinum NT types A, B and E with the surface of liposomes made of different lipid compositions was studied by photolabelling with a radioiodinated photoactive phosphatidylethanolamine analogue [^{125}I-dipalmitoyl (3,4-azidosalicylamido)phosphatidylethanolamine]. When the vesicles were made of negatively charged lipids (asolectin), each of these neurotoxic proteins was radioiodinated, thus providing evidence for their attachment to the membrane surface. The presence of gangliosides on liposome membranes enhanced fixation of the neurotoxic proteins to the lipid vesicle surface. Both the heavy and light chains of the NTs were involved in the attachment to the lipid bilayer surface. Each of the NTs tested here attached poorly to liposomes made of zwitterionic lipids (egg phosphatidylcholine), even when polysialogangliosides were present. The data suggest that the binding of NTs to their target neuronal cells involves negatively charged lipids and polysialogangliosides on the cell membrane.

4. FAST PROTEIN LIQUID CHROMATOGRAPHY OF NEUROTOXIN TYPES A, B AND E:

In order to derive the benefits of an automated modern chromatographic system the Fast Protein Liquid Chromatography was employed to: i) examine whether isolation of a NT from its complex can be made more efficient (operation time, yield and purity of NT); and ii) establish the chromatographic parameters of NT. Three antigenically different NTs (M_r ~150,000), classically distinguished only by specific antisera, were for the first time chromatographically resolved. Mixed NTs eluted from the Mono Q column in order of types E, A and B, and from the Mono S column as B, E and A. Types A and B NTs were successfully chromotographed on the cation exchanger Mono S column above their isoelectric points. Purification of types A and B NTs by automated liquid chromatography was also accomplished for the

first time. Types A, B and E NTs were purified by application on anion-exchanger Mono Q, followed by use of cation-exchanger Mono S column.

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